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Directionality and Coordination of Dehydration and Ring Formation during Biosynthesis of the Lantibiotic Nisin

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The lantibiotic nisin is a potent antimicrobial substance, which contains unusual lanthionine rings and dehydrated amino acid residues and is produced by *Lactococcus lactis*. Recently, the nisin biosynthetic machinery has been applied to introduce lanthionine rings in peptides other than nisin with potential therapeutic use. Due to difficulties in the isolation of the proposed synthetase complex (NisBTC), mechanistic information concerning the enzymatic biosynthesis of nisin is scarce. Here, we present the molecular characterization of a number of nisin mutants that affect ring formation. We have investigated in a systematic manner how these mutations influence dehydration events, which are performed enzymatically by the dehydratase NisB. Specific mutations that hampered ring formation allowed for the dehydration of serine residues that directly follow the rings and are normally unmodified. The combined information leads to the conclusion that 1) nisin biosynthesis is an organized directional process that starts at the N terminus of the molecule and continues toward the C terminus, and 2) NisB and NisC are alternating enzymes, whose activities follow one after another in a repetitive way. Thus, the dehydration and cyclization processes are not separated in time and space. On the basis of these results and previous knowledge, a working model for the sequence of events in the maturation of nisin is proposed.

Nisin is a lantibiotic produced by *Lactococcus lactis*, which has been known since 1928 (1, 2). This antimicrobial peptide is active against various Gram-positive bacteria and has attained commercial success as a food preservative (3). In addition to the wide industrial applications of nisin, it became also a model system to study various aspects of lantibiotic biosynthesis, regulation, and mode of action (2). Furthermore, recently, other applications of nisin have emerged. Its biosynthetic machinery can be successfully used to install dehydrated amino acids and lanthionine rings in peptides, which are either related or totally unrelated to nisin (4–11). This offers great opportunities to modulate the stability and activity of peptides that are used as therapeutics (8).

The post-translational modified nisin molecule is classified as a member of the Group A lantibiotics (12). Mature nisin contains 34 amino acids, three of which are posttranslationally modified, and five thioether rings that are enzymatically formed upon cyclization of five free cysteines and five dehydroamino acid residues (Fig. 1). These peculiar modifications, which are very rare in nature, give nisin its exceptional stability against proteolysis and contribute greatly to its antimicrobial activity.

Nisin is synthesized ribosomally as a 57-amino acid residue-long polypeptide. Subsequently, it is directed to a putative synthetase complex that probably consists of three different proteins that include the dehydratase NisB, responsible for dehydration of serines and threonines to dehydroalanines and dehydrobutyrines, respectively; the cyclase NisC, which forms (methyl) lanthionine bridges between cysteines and dehydroamino acids; and the ABC transporter NisT, which performs transport across the lipid bilayer by consuming ATP. Newly synthesized and modified prenisin is still antimicrobially inactive. Only upon cleavage of the leader sequence that encompasses the first 23 amino acids by the dedicated protease NisP, an active molecule is liberated.

Although there are data pointing to the existence of a synthetase complex that modifies nisin, such a complex has not been isolated so far. However, both NisB (13, 14) and NisC (13) were shown by specific antibody detection to localize at the cytoplasmic membrane, although some soluble signal was also detected. This localization gives NisBC the opportunity to interact with the transporter NisT, which is an integral membrane protein. Furthermore, co-immunoprecipitation and yeast two-hybrid studies suggested an interaction between members of the nisin modification machinery and nisin itself (13). The function of each member of the putative multimeric synthetase has been investigated *in vivo* by knock-out studies. It also has been demonstrated that subsequent steps in nisin biosynthesis can be performed separately. Dehydration, cyclization, and transport of the modified product were dissected *in vivo*, and also the dehydratase has been shown to perform enzymatic reactions without the presence of other members of the complex *in vivo* (7) although with very low efficiency. The cyclization activity of NisC was demonstrated *in vitro* (15), and the ABC transporter NisT was shown to be capable of transport of unmodified prenisin *in vivo* (10). Based on the available data, it is difficult to assess whether multimeric lanthionine complexes are indispensable for efficient nisin production and modification. However, *in vivo* localization studies and interaction experiments suggest that these proteins work in a concerted manner.

Here, we present data that indicates a strong coordination between members of the nisin modification machinery. The

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TABLE 1
***L. lactis* strain and plasmids used in the study**

	Characteristics	References
Strain		
NZ9000	*nisRK*	Ref. 24
Plasmids		
pIL3BTC	*nisBTC*, encodes for the nisin modification machinery	Ref. 9
pIL3BTCH331A	*nisBTC* with mutation H33iA in *nisC*	This study
pNZnisA-E3	*nisA*, encoding for nisin	Ref. 10
pNZE3nis 1	*nisA*, encoding for nisin, with S3A mutation	This study
pNZE3nis 2	*nisA*, encoding for nisin, with T8A mutation	This study
pNZE3nis 3	*nisA*, encoding for nisin, with T13A mutation	This study
pNZE3nis 4	*nisA*, encoding for nisin, with T23A mutation	This study
pNZE3nis 5	*nisA*, encoding for nisin, with T25A mutation	This study
pNZE3nis 1 + 4	*nisA*, encoding for nisin, with S3A and T23A mutations	This study
pNZE3nis 2 + 5	*nisA*, encoding for nisin, with T8A and T25A mutations	This study
pNZE3nis K12S	*nisA*, encoding for nisin, with K12S mutation	This study
pNZE3nis 2 K12S	*nisA*, encoding for nisin, with T8A and K12S mutations	This study
pNZE3nis N20S	*nisA*, encoding for nisin, with N20S mutation	This study
pNZE3nis 3 N20S	*nisA*, encoding for nisin, with T13A and N20S mutations	This study

analysis of sets of nisin mutants, where key residues that take part in ring formation as well as substitutions of residues that directly follow lanthionine structures, suggests a strong interdependency of dehydratase and cyclase activity. Moreover, the data indicate that these enzymes alternate during catalysis and that they are intertwined in time and space. Our data also suggest that nisin modification is an ordered process that proceeds consecutively from the N terminus of prenisin toward its C terminus. Based on the available literature data and the data presented here, we propose a model wherein nisin is being post-translationally modified in consecutive steps from its N terminus toward its C terminus.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—Table 1 shows all the strains and plasmids that were used in this study. Mutated versions of prenisin were overexpressed, modified, and secreted by means of a two-plasmid expression system, as described before (10). Briefly, *L. lactis* was used as a host for overexpression plasmids pNZnisA-E3, which encodes prenisin and pIL3BTC, which encodes the nisin modification and transport machinery. Cells were grown as described previously (16) at 30 °C in M17 medium (Difco) supplemented with 0.5% (w/v) glucose and antibiotics 5 μg/ml chloramphenicol and erythromycin (2 μg/ml) where appropriate.

Recombinant DNA Techniques—Standard genetic manipulations were essentially performed as described by Sambrook *et al.* (17). Plasmid pNZnisA-E3 (10) was received as a kind gift from BioMade Foundation Groningen and served as a template for round PCR in order to obtain point mutations. The round PCR method with 5′-phosphorylated primers was performed as described earlier (9). Plasmid isolation was performed by means of the plasmid DNA isolation kit (Roche Applied Science). Restriction analysis was performed with restriction enzymes from Fermentas. DNA ligation was performed with T4 DNA ligase (Fermentas), and round PCR amplification was done with Phusion DNA polymerase (Finnzymes).

Protein Expression and Purification—Antimicrobial peptides were purified as described before (9, 16). *L. lactis* NZ9000 carrying pIL3BTC and pNZnisA-E3 or its mutated variants was grown overnight in GM17 medium supplemented with 5 μg/ml chloramphenicol and erythromycin (2 μg/ml) in order to maintain the plasmids. In the morning, cultures were diluted 1:50 into a chemically defined medium supplemented with MOPS[4] buffer, containing appropriate antibiotics and 0.5 ng/ml nisin as an inducer. Bacteria were cultured at 30 °C for 24 h, and subsequently supernatant was collected by centrifugation. Supernatant, which contained secreted antimicrobial peptides, was filtered through a 0.2-μm filter and subjected to fast protein liquid chromatography. Supernatant was mixed 1:1 with a wash buffer, 50 mM lactic acid and applied to a HiTrap SP-Sepharose (GE Healthcare) column for cation exchange chromatography. Peptides were washed with 50 mM lactic acid and eluted with 50 mM lactic acid, 1 M NaCl. Fast protein liquid chromatography (FPLC) was conducted with an Akta purifier instrument (Amersham Biosciences).

Mass Spectrometry Analysis—In order to conduct mass spectrometric analysis of the produced peptides, we used crude supernatants or FPLC-purified fractions. Prior to the mass spectrometric analysis, samples were ZipTipped (C18 ZipTip; Millipore) essentially as described before (10, 16). In short, ZipTips were equilibrated with 100% acetonitrile and washed with 0.1% trifluoroacetic acid. Subsequently, the supernatant containing the peptides was mixed with 0.1% trifluoroacetic acid and applied to ZipTip. Bound peptides were washed with 0.2% trifluoroacetic acid and eluted with 50% acetonitrile and 0.1% trifluoroacetic acid. The eluent was mixed in a ratio of 1:1 with matrix (10 mg/ml α-cyano-4-hydroxycinnamic acid), and 1.5 μl was spotted on the target and allowed to dry. Mass spectra were recorded with a Voyager-DE Pro (Applied Biosystems) MALDI-TOF mass spectrometer in a linear positive mode (instrument settings: acceleration, 20,000 V; grid, 92%; guide wire, 0.1%; delay time, 100 ns). In order to increase the sensitivity, external calibration was applied with six different peptides (protein MALDI-mass spectrometry calibration kit; Sigma).

Growth Studies—Antimicrobial activity of FPLC-purified prenisin and its mutants was tested against a sensitive *L. lactis* strain, as described before (16). Briefly, strain NZ9000 contain-

[4] The abbreviations used are: MOPS, 4-morpholinepropanesulfonic acid; FPLC, fast protein liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.

ing pNZnisPT was used as an indicator. It is not only sensitive to nisin but also produces NisP, which is the prenisin-processing protease. The protease is used to cut prenisin mutants used in the experiment and liberate their antimicrobial active form. The minimal inhibitory concentration of an antimicrobial peptide was determined by growth in 96-well microtiter plates by a method similar to that described before (18). Briefly, overnight cultures of an indicator strain, NZ9000 containing pNGnisPT, were diluted into a fresh medium and grown to A_{660} of 0.5. Subsequently, cells were diluted 10 times, and 150-μl aliquots were mixed with 50 μl of GM17 medium supplemented with chloramphenicol (5 μg/ml), nisin (0.5 ng/ml) (necessary for expression of NisP), and various concentrations of antimicrobial peptides. Growth of cells was monitored at 650 nm every 10 min for 16 h by means of a multiscan photometer (Thermo Max microplate reader; Molecular Devices). The minimal inhibitory concentration was determined as the minimal concentration of an antimicrobial peptide that caused growth inhibition ($A_{660} <$ 0.2) at a time when the indicator strain grown in parallel (without any antimicrobial peptide) reached stationary phase.

RESULTS

Production, Modification, Secretion, and Purification of Nisin Mutants—It still remains to be elucidated whether enzymes that are responsible for nisin modification and secretion form a complex and work consecutively or if they work separately, allowing random order modifications. This in part relates to the fact that isolation of the complex and its biochemical analysis remains a challenging and still unsuccessful task. We have decided to try to analyze nisin modification enzymes *in vivo* from the viewpoint of their substrate. For this purpose, we have designed and produced a series of mutants and analyzed the capabilities of the dehydratase and cyclase enzymes to accept and modify specific residues. Moreover, we have investigated in detail the dehydration pattern of our mutants to determine whether the changes we introduced have local effects or whether they influence the modification reaction globally.

In order to achieve this goal, we used an established expression and modification system (4–10). Thus, *L. lactis* NZ9000 was used as a host for production, modification, and secretion of nisin and its mutants. In order to overexpress peptides of interest, we used a two-plasmid system, where pIL3BTC encodes for nisin modification and secretion enzymes, and pNZnisA-E3 encodes for wild type nisin under its native promoter (10). The latter plasmid was used as a template to produce nisin mutants, as described under "Experimental Procedures." We have prepared a series of mutants serving several different purposes that will be described in detail below. The peptides were overexpressed and subjected *in vivo* to the nisin modification enzymes and secreted. Next, we purified them by means of FPLC using a HiTrap SP-Sepharose (GE Healthcare) column for cation exchange chromatography. The mutants were well expressed, and we could purify sufficient quantities for further biochemical analysis (data not shown).

Dehydration Events Proceed Independently from One Another—Nisin contains three dehydrated amino acids and five thioether rings. Serines and threonines in the leader peptide, which is cleaved off by NisP, as well as the serine at position 29 in the mature peptide are never dehydrated. Furthermore, serine at position 33 sometimes escapes dehydration (Fig. 1). *In silico* analysis of lantibiotic structures did not reveal a conserved sequence motif that could encompass a modification signal. However, it was demonstrated that dehydratable serines and threonines are preferably flanked by hydrophobic rather than by hydrophilic amino acids (9). We have now investigated whether serines and threonines, which take part in ring formation, affect dehydration of other serines and threonines. New nisin variants that have serine or threonine residues substituted by alanine in either one of the five rings and two mutants containing double point mutations (namely in either rings A and D or rings B and E) were generated (Fig. 1). Fig. 2 shows examples of MALDI-TOF spectra, which were obtained after analysis of FPLC-purified and further zip-tipped peptides. Table 2 summarizes masses of the various peptides that we have analyzed. Prenisin showed two major peaks, the masses of which correspond to peptides being 8- or 7-fold dehydrated (Figs. 1 and 2 and Table 2). This is schematically depicted in Fig. 1, in which seven dehydrations are *shaded gray*, and serine 33, which sometimes escapes dehydration, is *shaded light gray*. Analysis of the five single mutants and the two double mutants showed that the maximal number of dehydrated residues was achieved in all cases. Mutants in rings A, B, and C in which single dehydratable residues were mutated to alanine showed peptide masses that corresponded to seven dehydrations. Mutations that concerned rings D and E resulted in a mixture of peptides where, besides the expected seven dehydrated residues, also other species containing additional dehydrated residues were found (Figs. 1 and 2 and Table 2). Similarly, the double mutants showed a number of different peptides, which included the predicted peaks of which the mass corresponded to six dehydrated residues (Table 2). Taken together, these data indicate that the mutational change of one dehydratable residue involved in ring formation does not significantly influence the subsequent dehydration reactions, except in the following case.

Two Intertwined Rings of Nisin Protect Ser^{29} against Dehydration—Careful analysis of the influence of mutations in the last two C-terminal rings showed that besides the expected peptide masses, which correspond to species with seven dehydrated residues at maximum, one other mass peak was observed. This peptide carries a mass that corresponds to a mutant species with eight dehydrated residues. The most likely candidate for another serine to be dehydrated is the serine at position 29 that normally is not dehydrated (Figs. 1 and 2 and Table 1). In order to rule out the possibility that this additional dehydrated residue is contributed by a residue in the leader sequence, we have analyzed species obtained by limited trypsinization of mutant nisin 4 and nisin 5 with the same result. The peak corresponding to eight dehydrated residues was found in both mutants (data not shown), suggesting that the only possible additional residue that can be dehydrated is actually serine 29.

The additional dehydration of residue 29 was only observed upon mutation of threonine 25, which precludes formation of ring E, and of threonine 23, which precludes formation of ring D (Fig. 1). We hypothesize that the direct presence of a lanthionine ring in front of serine 29 precludes its dehydration. Ring E

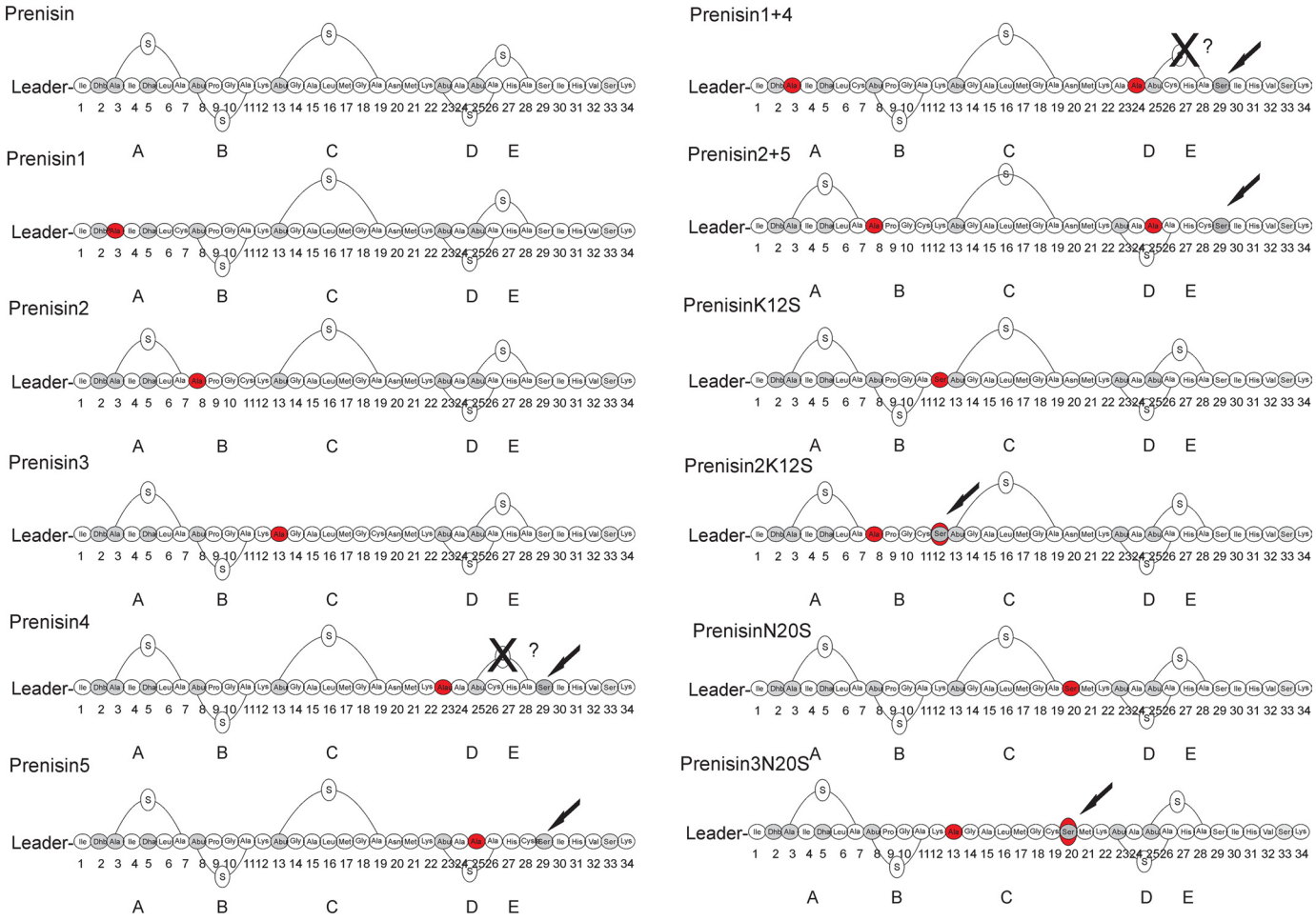

FIGURE 1. **Primary structure of prenisin and generated mutants.** Dehydrated residues are *shaded gray*; serine 33 sometimes escapes dehydration and is shaded *light gray*. Serine at position 29 is never dehydrated in wild type prenisin. The impact of mutations on the dehydration pattern of new prenisin species is schematically depicted. Mutated residues are indicated by *filled red circles*. Newly formed dehydrated residues are pointed to by a *black arrow*. *Letters A–E* correspond to the five consecutive lanthionine rings in nisin.

is adjacent to serine 29, and its absence results in dehydration of this residue. Similarly, lack of ring D also results in additional dehydration, although it is not an immediate neighbor of serine 29. Due to the close vicinity of this two rings and the fact that they are intertwined, lack of formation of one of them may negatively influence the formation of the other. This lack of ring formation in turn determines whether or not the serine 29 becomes dehydrated.

Dehydration and Cyclization Are Alternating Processes during Nisin Biosynthesis—We have investigated whether the ring protection phenomenon that we observed was restricted to the particular site within the peptide or is due to the direct vicinity of an N-terminally located lanthionine ring. To analyze other positions in the nisin molecule, four additional mutants were generated. We replaced the lysine residue that directly follows ring B by serine (K12S) and combined this mutation with a mutant that does not have ring B due to the mutation of threonine that forms the ring (prenisin 2 K12S) (Fig. 1). This allowed us to investigate whether an introduced serine behind ring B will be dehydrated and whether the presence/absence of ring B has influence on this reaction. Similarly, asparagine at position 20 was changed to serine (N20S), and this mutation was combined with a mutant lacking ring C (prenisin 3 N20S) (Fig. 1). These mutants were successfully overproduced in *L. lactis* harboring pIL3BTC, which encodes the nisin modification machinery and FPLC-purified for further analysis. Zip-tipped mutants were analyzed by mass spectrometry for their dehydration pattern (Fig. 3). An additional serine behind ring B (K12S) is dehydrated to only a very limited extent, and the major mass peak that was observed corresponds to a peptide with eight dehydrated residues, suggesting that the new serine 12 is largely protected by the presence of ring B against the action of the dehydratase NisB. Mutation of the residue following ring C (N20S) to the potentially dehydratable serine results in an unchanged dehydration pattern. The identified species carry eight and seven dehydrated residues, indicating that the newly installed serine at position 20 is not dehydrated. This, once again, points to the important role of lanthionine rings in the protection of these posterior serines at different positions within the propeptide against dehydration. If the hypothesized role of the ring protection is true, lack of the ring preceding either serine 12 or serine 20 should result in their full processing. This is exactly what we observed using the following constructs. Mutant prenisin 2 K12S and prenisin 3 N20S, have serines following ring B and ring C, respectively (Figs. 1 and 3). Moreover, in these mutants, dehydratable Ser and Thr residues

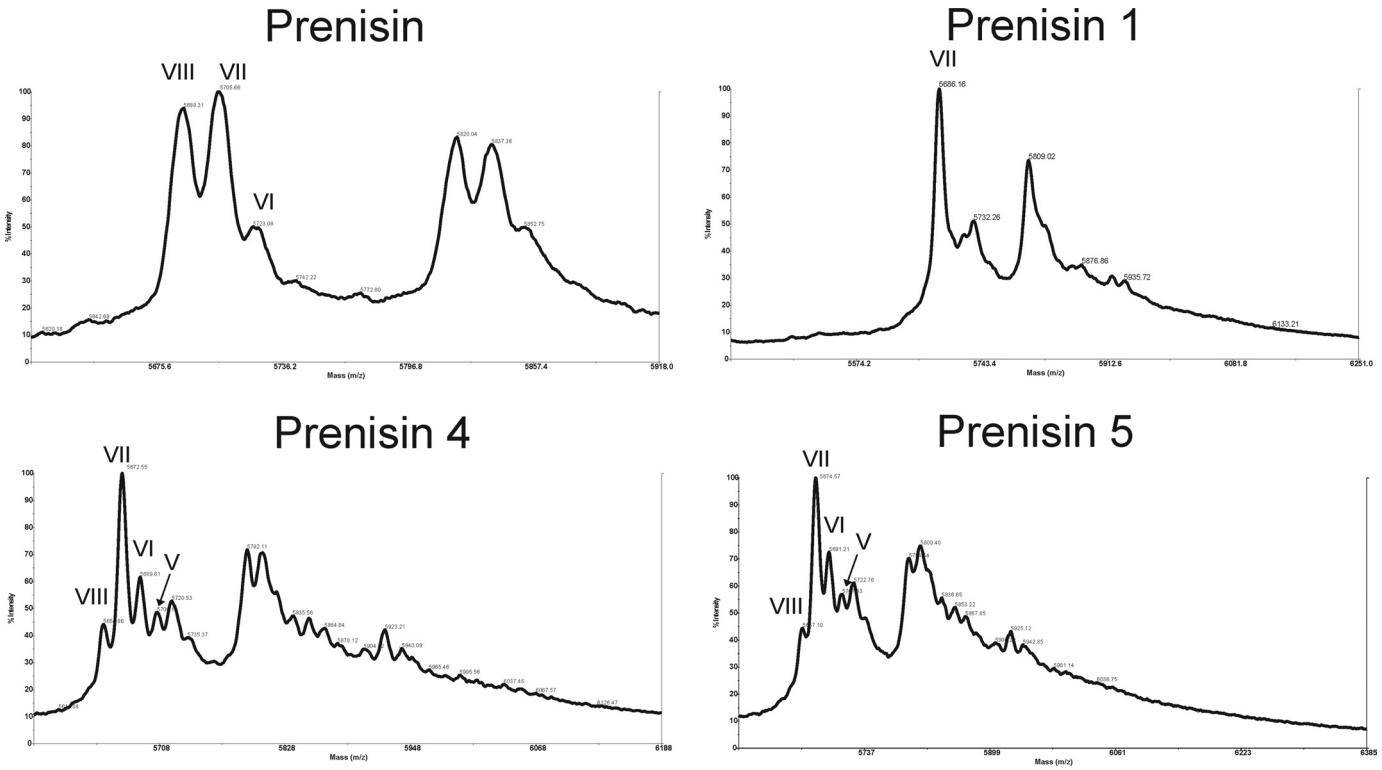

FIGURE 2. **Examples of mass spectra of prenisin and prenisin mutants produced by *L. lactis* NZ9000 harboring pIL3BTC plasmid required for expression of nisin modification machinery and various pNZnisA-E3 plasmids encoding for prenisin and its mutants.** Visualized are mass peaks corresponding to species of prenisin or its mutants without initial methionine (*L. lactis* usually removes initial methionine from synthesized peptides) with different numbers of dehydrated residues indicated by *roman numerals*. Other major peaks represent prenisin species containing the initial methionine with different degrees of dehydration (the dehydration pattern of peptides with and without initial methionine is identical), which were not labeled due to clarity.

that take part in formation in ring B and ring C were mutated to alanines. Notably, these mutants displayed a strong additional dehydration peak, which corresponded to eight dehydrated residues, suggesting that when the rings are missing, the serines at positions 12 and 20 become a substrate for NisB and become dehydrated (see Figs. 1 and 3 and Table 2). This finding suggests that the ring protection phenomenon, which was observed with serine 29, is not restricted to that part of the molecule but appears to be a general mechanistic feature of the dehydration reaction. Moreover, this phenomenon has important implications for the working modes of the dehydration and cyclization enzymes.

A NisC Catalytic Mutant Is Able to Efficiently Produce Ringless Nisin with an Extra Dehydrated Serine—We have demonstrated that the direct vicinity of the lanthionine rings has a profound effect on the ability of the dehydratase to modify neighboring dehydratable residues. Recently, a publication appeared showing that although NisB, NisT, and NisC have been shown to be active separately, the kinetics of the overall process is significantly altered (19). Clean deletions of each component of the transport/modification machinery showed that nisin production is only efficient when all members of the synthetase complex are present (19). Deletion of *nisC* resulted in a strong decrease in prenisin production, which suggests that the nisin biosynthesis is severely hampered. Mass spectrometric analysis of such produced and modified prenisins revealed a peptide species that carries either eight or seven dehydrated residues (*i.e.* similar to the wild type). This suggests that serine 29 was not dehydrated in the absence of NisC, due to the fact that lack of NisC may disturb the synthetase complex formation and influence strongly the kinetics and efficiency of the whole process. Therefore, we decided to analyze catalytically inactive mutants of NisC. We mutated histidine 331 to alanine in NisC using pIL3BTC as a template plasmid for a mutational change. This conserved histidine has been shown to play a role in the coordination of a zinc ion, and its change to alanine abolished cyclization activity of NisC but did not change its affinity for prenisin (15, 20). Production and secretion of nisin using the mutated version of pIL3BTC^{H331A} showed similar production levels as compared with those of the wild type two-plasmid system (data not shown). Moreover, mass spectrometry analysis of prenisin clearly indicated the presence of mass peaks that corresponded to a peptide that carries nine and eight dehydrated residues (Fig. 4*A* and Table 2). This indicates that the complex formation and the presence of NisC, but not its catalytic activity is important for the proper kinetics of nisin biosynthesis. Furthermore, catalytically inactive NisC did not install lanthionine rings, resulting in additional dehydration of serine 29. In order to exclude the possibility that this additional dehydration may reside in residues of the leader sequence, we digested prenisin with trypsin, which resulted in cleavage of the leader sequence. The obtained masses corresponded to the peptides carrying nine, eight, and seven dehydrated residues (Fig. 4*B*). This clearly shows that lack of the catalytic activity of NisC and lack of lanthi-

TABLE 2
Dehydration of prenisin and its mutants by NisB, analyzed by MALDI-TOF mass spectrometry

Peptide	No. of dehydratable residues	Dehydration extent	Mass ($M + H^+$) without Met1	
			Observed	Calculated
			Da	
Prenisin wild type	9	8	5688.31	5688
		7	5705.66	5706
		6	5723.08	5724
Prenisin 1	8	7	5686.16	5686
Prenisin 2	8	7	5675.91	5676
Prenisin 3	8	7	5676.5	5676
Prenisin 4	8	8	5654.86	5658
		7	5672.55	5676
		6	5689.61	5694
		5	5706.12	5712
Prenisin 5	8	8	5657.1	5658
		7	5674.57	5676
		6	5691.21	5694
		5	5707.83	5712
Prenisin 1 + 4	7	7	5660.42	5659
		6	5677.2	5677
Prenisin 2 + 5	7	7	5646.08	5646
		6	5662.82	5664
Prenisin K12S	10	9	5628.28	5629
		8	5645.06	5647
		7	5662.08	5665
Prenisin N20S	10	8	5661.4	5661
		7	5678.05	5679
Prenisin 2 K12S	9	8	5617.67	5617
		7	5633.45	5635
Prenisin 3 N20S	9	8	5631.42	5631
		7	5648.15	5649
Prenisin (produced by $NisC^{H331A}$)	9	9	5669.2	5670
		8	5686.53	5688
Nisin (produced by $NisC^{H331A}$ and trypsinated)	9	9	3333.92	3336
		8	3352.14	3354
		7	3371.17	3372
Prenisin K12S (produced by $NisC^{H331A}$ and trypsinated)	10	10	5612.39	5611
		9	5630.41	5629
		8	5647.29	5647
		7	5663.58	5665
Prenisin 2 K12S (produced by $NisC^{H331A}$ and trypsinated)	9	9	5600.04	5599
		8	5617.49	5617
		7	5633.98	5635
		6	5650.73	5653
Prenisin N20S (produced by $NisC^{H331A}$ and trypsinated)	10	10	5626.17	5625
		9	5643.61	5643
		8	5659.71	5661
		7	5677.34	5679
Prenisin 3 N20S (produced by $NisC^{H331A}$ and trypsinated)	9	9	5613.74	5613
		8	5630.41	5631
		7	5646.80	5649
		6	5663.60	5667

onine ring formation release protection of serine 29, which is now available for the dehydratase.

Furthermore, in order to investigate if the observed effects are specific to position 29 in the nisin molecule, we also studied the effects of the NisC catalytic mutant on nisin mutants that harbor additional potentially dehydratable residues at two different positions of the molecule. Nisin mutant K12S, which contains an additional serine following ring B and showed predominantly eight dehydrated residues when processed by wild type NisC, was subjected to maturation by the complex that contained $NisC^{H331A}$. The produced polypeptide contained a maximum of 10 dehydrations. This indicates that when the rings were not installed by the mutated NisC, additional serines, Ser^{29} and Ser^{12}, became dehydratable (Fig. 5*A*). Likewise, mutant 2 K12S, which harbors a mutation of a dehydratable residue that normally forms ring B when maturated by the wild type complex, shows eight dehydrations (Fig. 3). This indicates that Ser^{12} was accessible for NisB due to the absence of ring B, but Ser^{29} was protected by ring E. Modification of this mutant by the complex that contained the mutated NisC resulted, as expected, in nine dehydrations, indicating dehydration of all possible dehydratable residues in the molecule. Similarly, mutant N20S and mutant 3 N20S maturated by $NisBTC^{H331A}$ showed a maximum of 10 and nine dehydrations, respectively (Fig. 5*B*). When these two mutants were processed by the wild type complex (Fig. 3), maximally eight dehydrations were observed in both cases.

Antimicrobial Activity of the New Nisin Mutants—In order to study the influence of the mutations on antimicrobial activity of nisin, all mutants were purified using FPLC. The obtained prepeptides were processed by the NisP protease *in vivo* in order to cleave off the leader peptide and liberate the mature molecule. Activity analysis of the nisin mutants processed by NisP demonstrated that mutations in the residues that form lanthionine rings have a strong negative influence on the antimicrobial activity, which confirms the crucial role of the rings

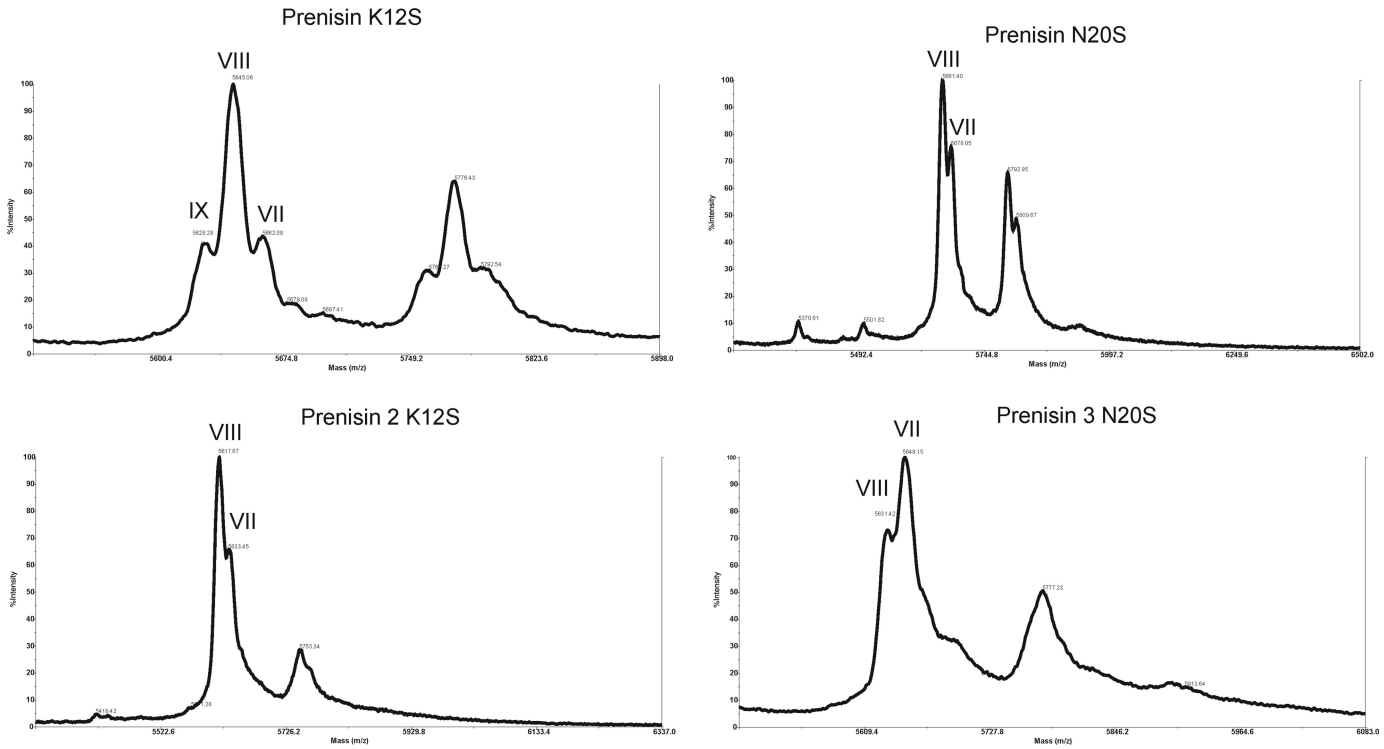

FIGURE 3. **Mass spectra of prenisin mutants produced by *L. lactis* NZ9000 harboring pIL3BTC plasmid required for expression of nisin modification machinery and various pNZnisA-E3 plasmids encoding for prenisin mutants.** Visualized are mass peaks corresponding to species of prenisin or its mutants without initial methionine (*L. lactis* usually removes initial methionine from synthesized peptides) with different numbers of dehydrated residues indicated by *roman numerals*. Other major peaks represent prenisin specious containing initial methionine with different degrees of dehydration (the dehydration pattern of peptides with and without initial methionine is identical), which were not labeled due to clarity.

for nisin activity (Table 3). As expected, the mutants that showed the lowest antimicrobial activity were those that were affecting rings that are important for binding to lipid II and form a "glovelike structure" (21). Substitution of residues 12 and 20 to serines did not significantly change the antimicrobial activity of nisin (Table 3).

DISCUSSION

Lantibiotics form a unique group of peptides produced by Gram-positive bacteria. Their uniqueness resides in the post-translationally introduced modifications, such as the unusual dehydrated amino acids and intramolecular thioether bridges they contain. Recent findings indicate that enzymes that modify lantibiotics can be used to modify other unrelated peptides (4–10, 16). This opens up an opportunity to use the nisin modification and secretion machinery to install these peculiar post-translationally modified residues in medically relevant peptides in order to modulate their activity (8). Although many data have been gathered up to now concerning the nisin biosynthetic enzymes, many mechanistic details concerning nisin modification reactions are still lacking. Some important questions are the following. Is there a sequential modification in an oriented way? How do NisB and NisC cooperate? In order to answer these questions, we should first consider the known features of nisin biosynthesis. First, nisin is synthesized as a 57-amino acid-long prepeptide, which can be divided into two main parts, the N-terminal leader part and the C-terminal structural part. The latter part will form the actual nisin, after modification and processing, which contains lanthionine rings and dehydrated amino acids. To have these incorporated, nisin has to meet the modification enzymes, which are hypothesized to work in a cooperative manner within a putative synthetase complex. Members of the complex, NisBTC, were found to localize at the cytoplasmic membrane (13), and multiple interactions between them and prenisin were demonstrated by co-immunoprecipitation and yeast two-hybrid screens (13). However, it was demonstrated that enzymes that form the putative complex can also work independently from each other. It was shown that NisB can dehydrate prenisin even if other modification enzymes are not present (7). Moreover, the ABC transporter NisT, which transports modified nisin, can also transport the unmodified prepeptide without the presence of NisBC (10). Finally, purified NisC has been shown to perform the cyclization reaction *in vitro* (15). Notably, a recent publication by van den Berg van Saparoea *et al.* (19) shows that lack of one of the components of lanthionine synthetase has a severe negative impact on the kinetics of the whole process. This indicates that the nisin modification and transport reaction is a concerted action of several distinct enzymes/proteins.

The N-terminal part of prenisin, the so-called leader sequence, has been shown to be important in recognition and binding to members of the synthetase complex (7, 13, 15). Nisin, when reaching the modification machinery, needs to be first dehydrated in order to be subsequently cyclized. The dehydration of serines and threonines to dehydroalanines and dehydrobutyrines, respectively, is performed by NisB (7, 22, 23). The cyclization of a dehydrated residue with free cysteines is per-

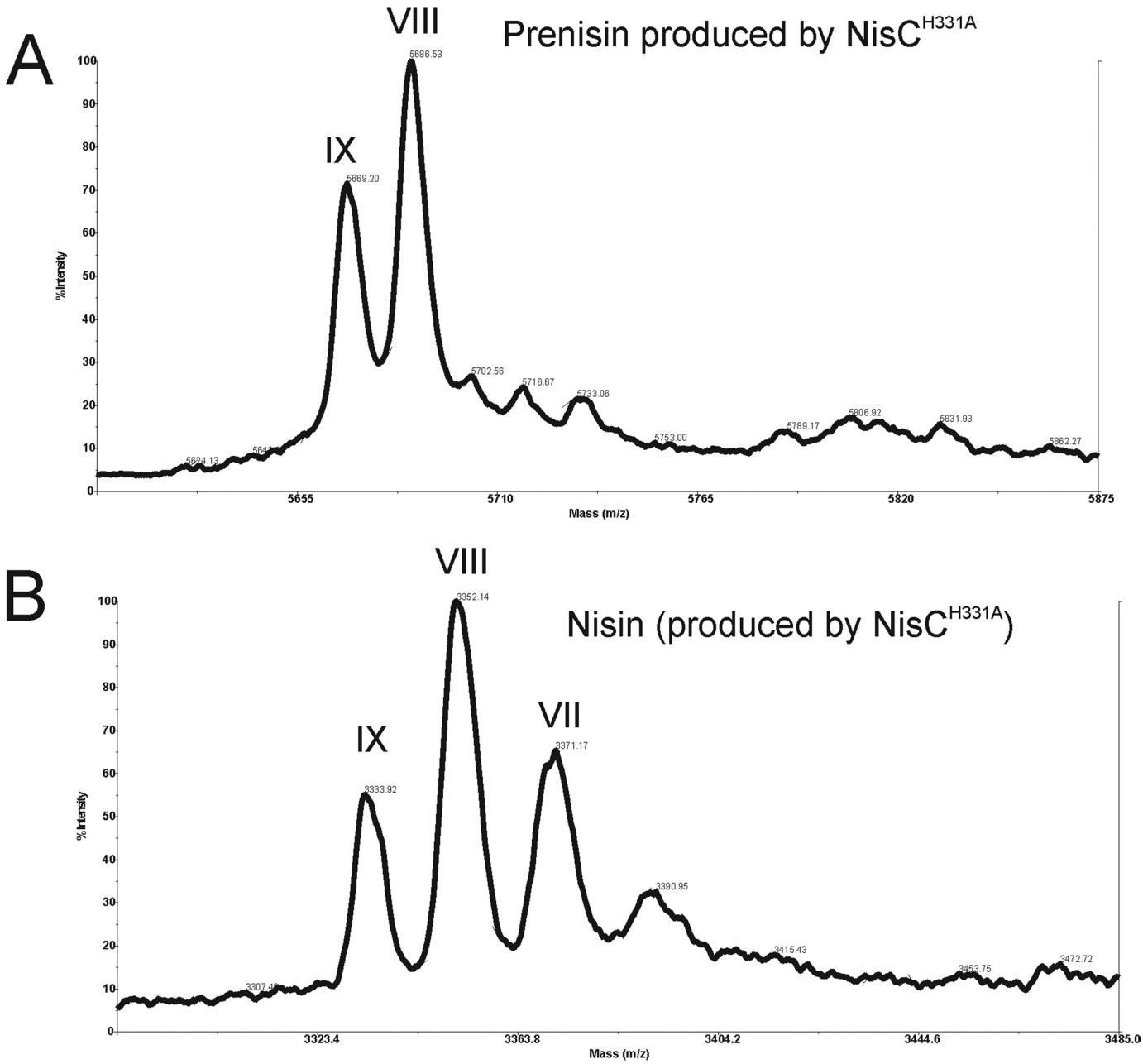

FIGURE 4. **Mass spectra of prenisin produced by *L. lactis* NZ9000 harboring pIL3BTC^{H331A} plasmid required for expression of nisin modification machinery, with functionally truncated NisC and pNZnisA-E3 plasmids encoding for prenisin.** *A*, two major mass peaks corresponding to prenisin (without initial methionine) dehydrated 8- and 9-fold. *B*, two major mass peaks corresponding to nisin (without initial methionine), dehydrated 8- and 9-fold.

formed by the cyclase NisC (10, 22, 23). Recently, NisC was reconstituted *in vitro* and crystallized (15). *In vitro* reconstitution of NisC gave a great insight into the molecular details of the cyclization reaction. A number of conserved residues were demonstrated to be important for binding of a zinc ion and for catalysis by a crystallographic study (15), which was corroborated by biochemical analysis (20). Furthermore, analysis of crystallographic data suggested the presence of a cleft in which the nisin leader could potentially bind (15). This binding groove is situated next to the catalytic site of NisC.

It has been demonstrated that upon fusion of the leader of nisin with peptides, which are entirely unrelated to the nisin structural part, dehydration, cyclization, and secretion of such chimeras is still possible (4–8, 10, 16). However, is there a limit to the wide substrate specificity of these enzymes? *In silico* analysis of a number of lantibiotics did not reveal any strong modification signals (9). It was predicted that hydrophobic residues flanking dehydratable amino acids are preferred over the hydrophilic ones. This was validated experimentally by analysis of the dehydration patterns of a variety of short peptides, specially designed for that purpose (9). Furthermore, it was investigated whether the distance at which serines and threonines are located from the leader sequence is important for the modification reaction to occur. The relative location also did not turn out to be a prerequisite for the dehydration reaction to take place (16).

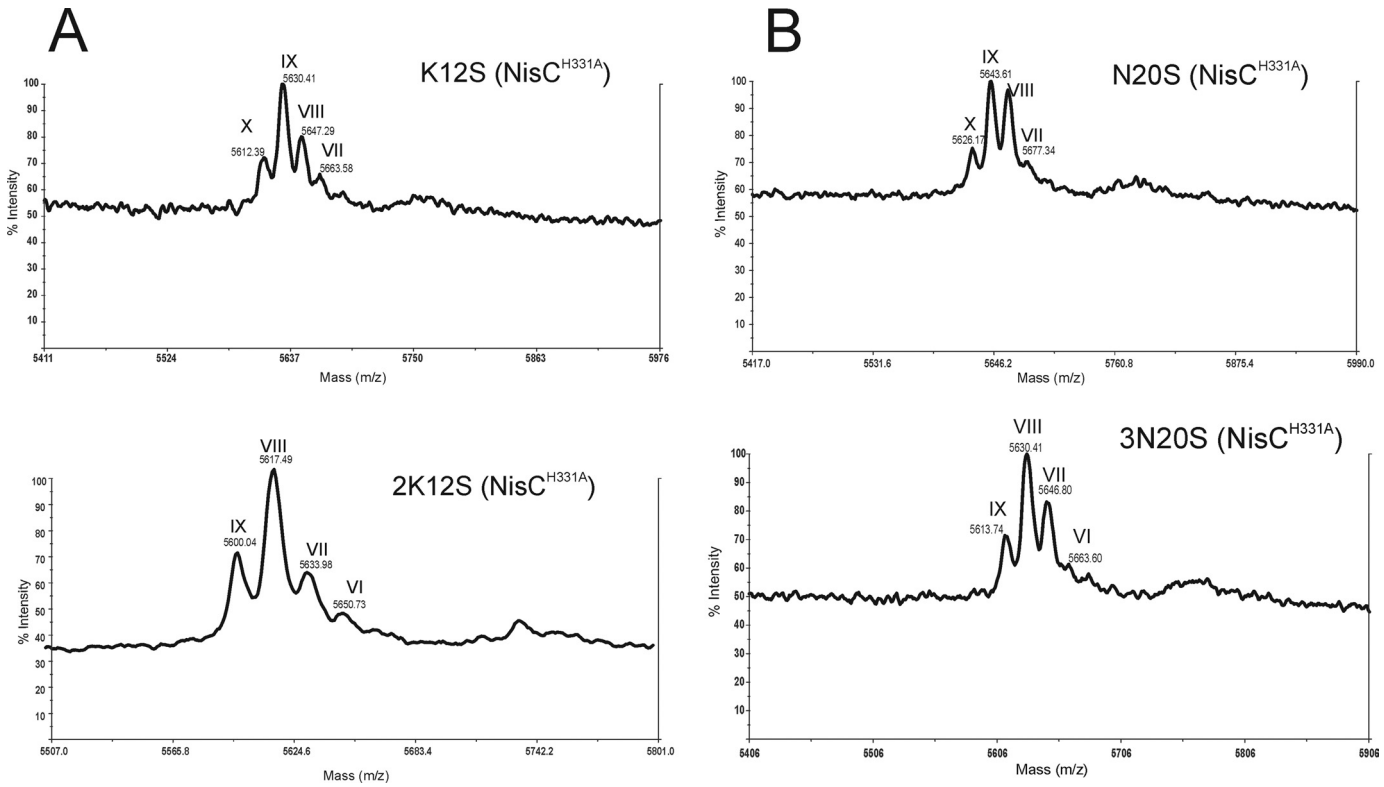

FIGURE 5. **Mass spectra of prenisin mutants produced by *L. lactis* NZ9000 harboring the pIL3BTC^{H331A} plasmid required for expression of the nisin modification machinery, with functionally truncated NisC and derivatives of pNZnisA-E3 plasmids encoding for prenisin mutants.** Shown are major mass peaks (without initial methionine) obtained for various mutants. *Panel A* shows mass peaks for mutant K12S, and *panel B* shows mass peaks for mutant N20S.

TABLE 3
Biological activity of (mutant) prenisin species

Antimicrobial	*L. lactis* NZ9000 pNGNisPT (indicator strain), minimal inhibitory concentration
	ng/ml
Nisin WT	25
Nisin 1	4000
Nisin 2	>4000
Nisin 3	>4000
Nisin 4	4000
Nisin 5	4000
Nisin 1 + 4	>4000
Nisin 2 + 5	>4000
Nisin K12S	25
Nisin N20S	25
Nisin 2 K12S	1000
Nisin 3 N20S	500

Prenisin as well as its modification enzymes were shown to interact with each other in different combinations. The nisin leader has been shown to be indispensable for dehydration, cyclization, and transport to occur jointly and separately. Presumably, the first step of nisin processing will be recognition and binding of the substrate by a nisin modifying enzyme. However, which of the enzymes will first bind prenisin? Or, alternatively, will it be bound at the interface between them? Will the dehydration reactions, which need to be performed before the cyclization, be completed before the cyclization by NisC starts? Are NisB- and NisC-catalyzed reactions separated in time and space? Finally, is the nisin modification reaction an ordered and directional process, or is it random? If this is a processive reaction, at which end of the molecule does it start?

Here, we present the analysis of a number of specifically designed nisin mutants, which suggests that the nisin post-translational modification reaction is a highly ordered and directional process that starts at the N terminus and proceeds to the C terminus. Furthermore, on the basis of collision-induced dissociation precursor analysis at different stages of modification, it has been suggested that modification reactions for HalM2 as well as LctM are likely to proceed from the N-terminal to C-terminal end (25). This notion is completely in line with our current findings and supports the model we provide in Fig. 6. Moreover, *in vivo* it appears that the nisin-modifying enzymes work in a concerted manner in order to efficiently modify and secrete their substrate. The evidence is outlined below.

We have demonstrated that the dehydration reaction of one dehydratable residue does not interfere or influence other dehydration events in the process, suggesting that the product of one dehydration reaction is not interlinked with another step in the process. Further, we noticed that the absence of ring E has a great influence on the dehydration of the neighboring serine 29. The same is true when ring D is mutated. Due to the fact that rings D and E are intertwined, the absence of ring D may preclude formation of ring E. Therefore, mutations in either ring D or E will result in lack of the ring that usually neighbors Ser^{29}. Interestingly, this residue, which normally is never dehydrated, becomes dehydrated in the mutants lacking ring E. This indicates that Ser^{29} could be protected against dehydration by ring E when present in nisin. In order to further investigate this hypothesis for other parts of the nisin molecule, we have designed and produced specific additional mutants. These

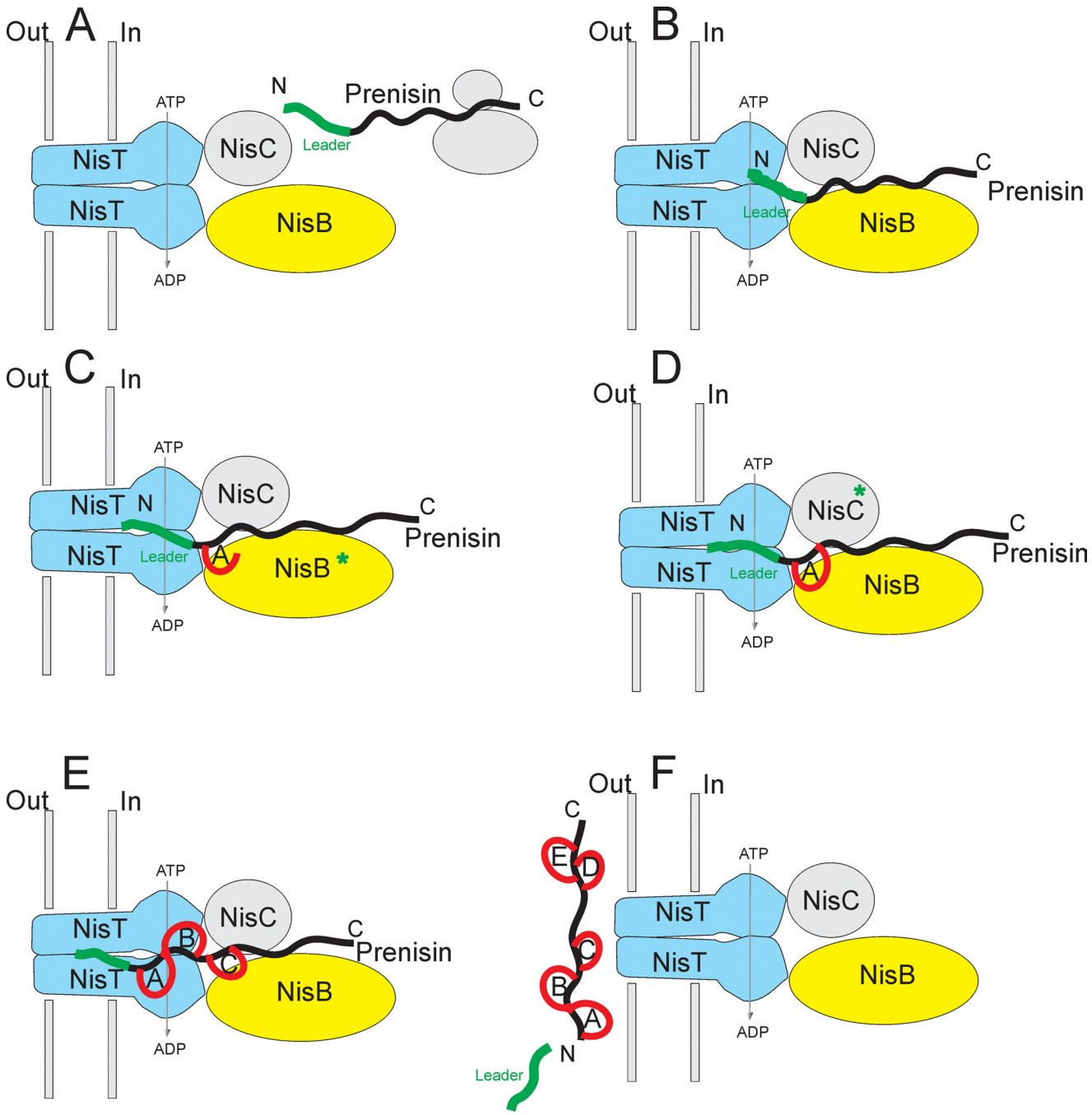

FIGURE 6. **Working model of nisin biosynthesis.** *A*, prenisin is synthesized ribosomally. *B*, prenisin leader is recognized and bound by lanthionine synthetase NisBTC. *C–E*, nisin is pulled, possibly by the transporter NisT, through the active site of NisB and NisC. NisC and NisB alternate in order to install lanthionine rings. First NisB (as indicated schematically by an *asterisk*) dehydrates available serines and threonines (*C*), and then NisC (indicated by an *asterisk*) cyclizes the dehydrated residue with an appropriate cysteine (*D*). *E*, subsequently, prenisin is pulled, possibly by NisT, and new rings can be installed consecutively. *F*, next, modified prenisin is transported across the lipid bilayer by NisT. In wild type, the precursor is then cleaved by NisP, yielding the active antimicrobial peptide.

mutants had a serine introduced behind either ring B or C, in combination with either the presence or absence of these particular rings. Similarly to Ser[29], the mutant with substitution N20S was only dehydrated when the preceding ring was absent. In case of the K12S substitution, the ring protection was partial; a minor mass peak was detected, suggesting that even if ring B is present, a small portion of the Ser[12] residue can become dehydrated. However, in the absence of ring B, Ser[12] was converted much more efficiently to dehydroalanine.

These data indicate that prenisin is probably bound by both modification enzymes and is being processed in a processive and concerted way, such that the modification process starts at the N terminus and finishes at the C terminus. When this would not be the case, much more dehydrated Ser would be encountered behind rings, which would then be formed after the dehydration event further downstream. The dehydratase NisB and cyclase NisC probably work in an alternating fashion, and the substrate is most likely not released during catalysis. First,

dehydration must occur, and then the dehydrated residue is cyclized to a free cysteine by NisC. At the very N-terminal end, a number of residues (at positions 2, 3, 5, and 8) are dehydrated probably before cyclization occurs. This includes Thr^8, which normally flanks ring A and is dehydrated. It is well known that threonines are more easily dehydrated than serines. However, when we substituted K12S, most of the product was not dehydrated. Furthermore, when the K12S mutation was combined with the ring B-disabling mutation, the new serine at position 12 became dehydrated. This suggests that before NisB reaches serine 12, NisC already has installed ring B, which probably causes a steric hindrance to the dehydratase action. Similarly, behind ring C and E, dehydration of a serine is only possible when the preceding rings are disabled. This suggests that nisin modification is an N to C terminus ordered process, where N-terminal residues are first dehydrated and then cyclized with appropriate cysteines before the reaction will proceed to the next dehydratable residue. Processivity and directionality of this reaction suggests that prenisin moves through the active site of an NisB and NisC complex or alternatively that these two enzymes physically move over the prenisin template. However, since we have not observed a cooperative link between the separate dehydration events, NisC was shown not to require ATP for cyclization, and NisB does not contain any obvious motifs in its primary amino acid structure that could bind and utilize high energy substrates such as ATP, it is tempting to speculate that one of the candidates that could physically pull the substrate through the active site of the lanthionine complex at the expense of ATP would be the NisT ABC transporter. This idea is supported by the observation that NisBT is known to have significantly lower modification activity without the presence of NisT. In another study, channeling interactions between members of the nisin modification machinery were indeed suggested (19).

Based on the available literature and the data shown here, we propose a new working model for nisin posttranslational modifications. Fig. 6 schematically depicts subsequent steps during nisin posttranslational modifications. Prenisin is synthesized ribosomally (Fig. 6*A*). It leaves the ribosome and is recognized and bound by lanthionine synthetase NisBTC at the cytoplasmic membrane. The first recognition site of the leader is still unknown and may involve each of the factors NisBTC (Fig. 6*B*). Subsequently, nisin is pulled, possibly by transporter NisT, through the active site of NisB and NisC. Whether NisB and NisC are in an oligomeric complex is still uncertain. Concomitantly, nisin is first modified by NisB (Fig. 6*C*), and subsequently NisC cyclizes newly formed dehydrated residues in the right position to free cysteines (Fig. 6*D*). This process proceeds from the N terminus of the nisin peptide to its C-terminal end in consecutive steps where cyclase and dehydratase alternate in catalysis (Fig. 6*E*). Next, fully modified nisin is completely transported by NisT to the outside of the cell (Fig. 6*F*).

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